11-28-97

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Office of the Deputy Assistant Commissioner for Patent Policy and Projects
REDDY et al	)	
Patent No.: 5,156,957	)	
Issued: October 20, 1992	)	November 28, 1997
For: FOLLICLE STIMULATING HORMONE	)	Atty.Docket: REDDY.EXT

APPLICATION FOR EXTENSION OF PATENT TERM

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

In accordance with 35 USC 156, patentee, Genzyme Corporation, through the undersigned attorney, hereby applies for extension of the term of the above-identified patent. Following is the information required by 37 C.F.R. §1.740.

(a)(1) The approved product is recombinant human follicle stimulating hormone (rhFSH). rhFSH consists of two non-covalently linked, non-identical protein components designated as the alpha- and beta-subunits. The alpha-subunit is composed of 92 amino acids carrying two carbohydrate moieties linked to Asn-52 and Asn-78. The beta-subunit is composed of 111 amino acids carrying two carbohydrate moieties linked to Asn-7 and Asn-24. The full amino acid sequences of the alpha-and beta-subunits of rhFSH, as determined by DNA sequencing of the cDNA and by direct sequencing of the protein subunits, are as follows:

Alpha-subunit:

Ala	Pro	Asp	Val	Gln	Asp	Cys	Pro	Glu	Cys 10
Thr	Leu	Gln	Glu	Asn	Pro	Phe	Phe	Ser	Gln 20
Pro	Gly	Ala	Pro	Ile	Leu	Gln	Cys	Met	Gly 30
Cys	Cys	Phe	Ser	Arg	Ala	Tyr	Pro	Thr	Pro 40

Leu	Arg	Ser	Lys	Lys	Thr	Met	Leu	Val	Gln	50
Lys	Asn	Val	Thr	Ser	Glu	Ser	Thr	Cys	Cys	60
Val	Ala	Lys	Ser	Tyr	Asn	Arg	Val	Thr	Val	70
Met	Gly	Gly	Phe	Lys	Val	Glu	Asn	His	Thr	80
Ala	Cys	His	Cys	Ser	Thr	Cys	Tyr	Tyr	His	90
Lys	Ser									92

Beta-subunit:

Asn	Ser	Cys	Glu	Leu	Thr	Asn	Ile	Thr	Ile	10
Ala	Ile	Glu	Lys	Glu	Glu	Cys	Arg	Phe	Cys	20
Ile	Ser	Ile	Asn	Thr	Thr	Trp	Cys	Ala	Gly	30
Tyr	Cys	Tyr	Thr	Arg	Asp	Leu	Val	Tyr	Lys	40
Asp	Pro	Ala	Arg	Pro	Lys	Ile	Gln	Lys	Thr	50
Cys	Thr	Phe	Lys	Glu	Leu	Val	Tyr	Glu	Thr	60
Val	Arg	Val	Pro	Gly	Cys	Ala	His	His	Ala	70
Asp	Ser	Leu	Tyr	Thr	Tyr	Pro	Val	Ala	Thr	80
Gln	Cys	His	Cys	Gly	Lys	Cys	Asp	Ser	Asp	90
Ser	Thr	Asp	Cys	Thr	Val	Arg	Gly	Leu	Gly	100
Pro	Ser	Tyr	Cys	Ser	Phe	Gly	Glu	Met	Lys	110
Glu										111

Asn - N-glycosylation sites

(a)(2) The product was approved under Section 505(b) of the Federal Food, Drug and Cosmetic Act (21 USC 355(b)).

(a)(3) The product received permission for commercial marketing or use under Section 505(b) of the Federal Food, Drug and Cosmetic Act on September 29, 1997.

(a)(4) As the present product is a human biological product and not a drug product (as those terms are used in the Federal Food, Drug and Cosmetic Act and the Public Health Service Act), 37 C.F.R. §1.740(a)(4) is not applicable.

(a)(5) The present application is being submitted within the 60 day period permitted for submission pursuant to 37 C.F.R. §1.720(f). The last date on which the application could be submitted is November 28, 1997, which is 60 days following the date of the FDA approval letter of September 29, 1997.

(a)(6) The patent for which an extension is being sought is U.S. patent 5,156,957 of which the inventors are Vemuri B. Reddy, Nancy Hsiung, Anton K. Beck and Edward G. Berstine. The date of issue was October 20, 1992, and the date of expiration is May 8, 2007. The terminal portion of the patent subsequent to May 8, 2007, has been disclaimed.

(a)(7) A copy of patent 5,156,957 is attached hereto as Exhibit A, including the entire specification (including claims and drawings).

(a)(8) Attached hereto as Exhibit B is a copy of the terminal disclaimer which was filed in the application which issued as patent 5,156,957 on July 17, 1991. Also attached hereto as Exhibit C is a copy of the receipt of maintenance fee payment establishing that the first maintenance fee was timely paid with respect to this patent.

(a)(9) The patent claims a method of manufacturing the approved product. Attached hereto as Exhibit D is a copy of Section 3.1 (page 45) from the January 15, 1997, amendment which was submitted during the FDA approval process. This section indicates that all of the information provided at pages 71-213 of the New Drug Application submitted on September 16, 1993, remains fully applicable. Attached hereto as Exhibit E are sections 3.1.1 and 3.1.2 (pages 71-84) of the September 16, 1993, original NDA submitted during the FDA approval process. These documents show the manufacturing process of the approved product. This manufacturing process of the approved product falls within the scope of claim 9 of the '957 patent. The following showing

demonstrates the manner in which this claim reads on the method of manufacturing the approved product.

Patent Claims	Approved Product
9. A method for producing the biologically active human fertility hormone FSH comprising	See the paragraph bridging pages 82 and 83 of Exhibit E which shows that the cell line produces 4,500-94,000 IU/l of FSH. As the FSH can be measured in international units, it must be biologically active. That it is human FSH is evident from page 72 which explains that the alpha- and beta-subunits were obtained from human genes obtained from a human genomic library.
culturing host mammalian cells in accordance with claim 1.	Note Section 3.1 on page 45 of Exhibit D which indicates that the production line is a 50 L cell culture process. Thus, the product is produced by culturing cells. That the cells being cultured are "host mammalian cells in accordance with claim 1" will be established by the following analysis of claim 1.
1. A mammalian cell comprising	Page 71 of Exhibit E shows that the host cell line used for the production of recombinant human follicle stimulating hormone is an anchorage dependent Chinese hamster ovary (CHO) cell line. As hamsters are mammals, these are clearly mammalian cells.
a transformed cell transformed by at least a first expression vector,	Page 71 of Exhibit E states in Section 3.1 that the CHO cells were cotransfected with two plasmids. The third paragraph on the same page indicates that the first plasmid is made by inserting the alpha-hFSH genomic fragment into a specific "expression vector". Thus, the transformed cell is transformed by at least a first expression vector.

said transformed cell being capable of producing a biologically active heterodimeric human fertility hormone comprised of an alpha-subunit and a beta-subunit, each said subunit being encoded in nature by a distinct mRNA, said hormone being human FSH,	See the discussion above with respect to claim 9 which establishes that the expression product of the transformed cell is a biologically active human FSH which is well-known to inherently contain an alpha-subunit and a beta-subunit, each being encoded in nature by a distinct mRNA. Note the discussion at pages 72-77 of Exhibit E relating to the isolation of separate alpha- and beta-hFSH genes.
the alpha-subunit of said hormone being encoded by said first expression vector and	As indicated hereinabove on page 71 of Exhibit E, the CHO cells were cotransfected with two plasmids, the former possessing the alpha-hFSH gene and the latter the beta-hFSH gene. As further explained above, the third paragraph of page 71 of Exhibit E establishes that the alpha-subunit was inserted into a specific expression vector for the purpose of making such a plasmid.
the beta-subunit of said hormone being encoded by said first expression vector or by a second expression vector by which said transformed cell is also transformed,	The cells cultured to make the approved product contain two expression vectors. The third paragraph of page 71 of Exhibit E explains that the second expression vector contains the beta-hFSH gene. The first paragraph on that page explains that the CHO cells are cotransfected with both expression vectors.
or a progeny of such transformed cell containing the genetic information imparted by said vector or vectors.	The cells actually being cultured are obviously progeny of the original transformed cells. That rhFSH is produced establishes that the progeny contains the genetic information imparted by the two expression vectors.

(a)(10)(i) The effective date of the Investigational New Drug (IND) application was February 26, 1992, and was assigned IND number 38,712. The New Drug Application was initially reviewed by the FDA on September 16, 1993, and received the reference number NDA 20-378. The NDA was approved on September 29, 1997.

(a)(11) The following is a brief description of significant activities undertaken by the marketing applicant during the applicable review period:

IND submitted	January 24, 1992
IND received by FDA	January 27, 1992
NDA submitted	September 15, 1993
NDA received by FDA	September 16, 1993
Amendments submitted	October 26, 1993 January 14, 1994 March 15, 1994 March 24, 1994 March 28, 1994 March 31, 1994 April 6, 1994 April 21, 1994 May 20, 1994 May 31, 1994 June 2, 1994 June 14, 1994
FDA not approvable letter	September 13, 1994
Meeting at FDA during which proposed responses to the not approvable letter were discussed	November 20, 1996
Amendment in response to not approvable letter submitted	January 15, 1997
FDA approvable letter	July 16, 1997
Submissions in response to approvable letter	July 17, 1997 July 21, 1997 July 22, 1997 July 29, 1997 August 13, 1997 August 19, 1997 August 29, 1997 September 17, 1997 September 24, 1997 September 26, 1997 (two) September 29, 1997
NDA approved	September 29, 1997

(a)(12) Applicant is of the opinion that patent 5,156,957 is eligible for patent term extension. Applicant claims a length of extension of 1,605 days, which will extend the patent through September 29, 2011. The length of extension was determined as follows using the following dates and time periods as set forth in 37 C.F.R. §1.775:

(c)(1)	02/26/92 through 09/16/93	= 599
(c)(2)	09/16/93 through 09/29/97	= 1475
(c)	(c)(1) + (c)(2)	= 2074
(d)(1)(i)	02/26/92 through 10/20/92	= 268
(d)(1)(ii)	not known	= 0
(d)(1)(iii)	((c)(1) - (d)(1)(i)) ÷ 2	= 165
(d)(1)	(c) - (d)(1)(i) - (d)(1)(ii) - (d)(1)(iii)	= 1641
(d)(2)	05/08/07 + (d)(1)	= 11/04/11
(d)(3)	09/29/97 + 14 years	= 09/29/11
(d)(4)	earliest of (d)(2) and (d)(3)	= 09/29/11
(d)(5)(i)	05/08/07 + 5 years	= 05/08/12
(d)(5)(ii)	earliest of (d)(4) and (d)(5)(i)	= 09/29/11
(d)	(d)(5)(ii)	= 09/29/11

(a)(13) Applicant acknowledges a duty to disclose to the Commissioner of Patents and Trademarks and the Secretary of Health and Human Services or the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought.

(a)(14) Attached hereto is a check in the amount of $1,120.00 in accordance with 37 C.F.R. §1.20(j)(1) for receiving and acting upon the application for extension.

(a)(15) The name, address and telephone number of the person to whom inquiries and correspondence relating to the present application are to be directed is as follows:

Roger L. Browdy
BROWDY AND NEIMARK
419 Seventh Street, N.W.
Suite 300
Washington, D.C. 20004-2299

Telephone: 202-628-5197
Facsimile: 202-737-3528.

(a)(16) The undersigned certifies that attached hereto is a duplicate of all of the present application papers.

(a)(17) I, the undersigned Roger L. Browdy, hereby declare and state that I am a patent attorney authorized to practice before the Patent and Trademark Office and have general authority from Genzyme Corporation, the owner of patent 5,156,957 to act on their behalf in patent matters relating to patent 5,156,957. I have reviewed and understand the contents of the foregoing application being submitted pursuant to 37 C.F.R. §1.740. I believe that the patent is subject to extension pursuant to §1.710. I believe that an extension of the length claimed, subject to any reduction caused by a determination by the Secretary of Health and Human Services under 35 USC 156(d)(2)(B) that applicant did not act with due diligence, is justified under 35 USC 156 and the applicable regulations. I believe that the patent for which the extension is being sought meets the conditions for extension of the term of a patent as set forth in 37 C.F.R. §1.720.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.

Attorneys for Patent Owner,

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US005156957A

United States Patent [19]

Reddy et al.

[11] Patent Number: 5,156,957

[45] Date of Patent: * Oct. 20, 1992

[54] **FOLLICLE STIMULATING HORMONE**

[75] Inventors: **Vemuri B. Reddy**, Framingham; Nancy Hsiung, Wellesley; **Anton K. Beck**, Chesnut Hill; **Edward G. Berstine**, Boston, all of Mass.

[73] Assignee: **Genzyme Corporation**, Boston, Mass.

[*] Notice: The portion of the term of this patent subsequent to May 8, 2007 has been disclaimed.

[21] Appl. No.: **323,665**

[22] Filed: **Apr. 24, 1989**

Related U.S. Application Data

[63] Continuation of Ser. No. 696,647, Jan. 30, 1985, Pat. No. 4,923,805, which is a continuation-in-part of Ser. No. 548,228, Nov. 2, 1983, Pat. No. 4,840,896.

[51] **Int. Cl.**[5] **C12P 21/06**; C12P 21/02; C12N 5/00; C07K 3/00

[52] **U.S. Cl.** **435/69.4**; 435/240.2; 435/320.1; 435/172.3; 435/70.1; 530/395

[58] **Field of Search** 435/69.1, 69.4, 172.3, 435/210.2, 320, 13, 66, 70

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,840,896	6/1989	Reddy et al.	435/68
4,923,805	5/1990	Reddy et al.	435/69.4

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Kaetzel, D. et al. (5 May 1988) J. Biol. Chem. vol. 263: 6344-6351 Methotrexate-Induced Amplification of the Bovine Lutropin Genes in Chinese Hamster Ovary Cells. Relative Concentration of the Alpha and Beta Subunits Determines the Extent of Heterodimer Assembly.

Kaetzel, D. et al. (Nov. 1985) Proc. Natl. Acad. Sci. USA, vol. 82: 7820-7283 Expression of Biologically Active Bovine Luteinizing Hormone in Chinese Hamster Ovary Cells.

Chemical Abstracts, vol. 109, No. 7 issued 1988, 14 Aug. (Columbus, Ohio, USA), Kato, Y. "Cloning and DNA Sequence Analysis of the cDNA for the Precursor of Porcine Follicle Stimulating Hormone Beta Subunit" See p. 182, col. 2, Abstract No. 49432a, Mol. Cell. Endocrinol (1988) 55: 107-12.

Primary Examiner—Jacqueline Stone
Assistant Examiner—Gian Wang

[57] **ABSTRACT**

Biologically active heterodimeric human FSH composed of an alpha subunit and a beta subunit, each subunit being synthesized by a cell having an expression vector containing heterologous DNA encoding the subunit.

10 Claims, 4 Drawing Sheets

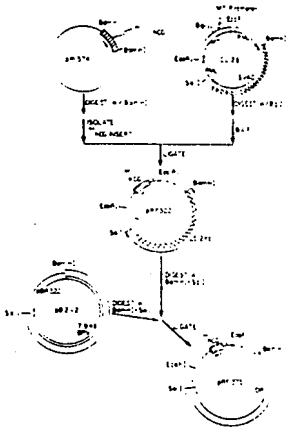
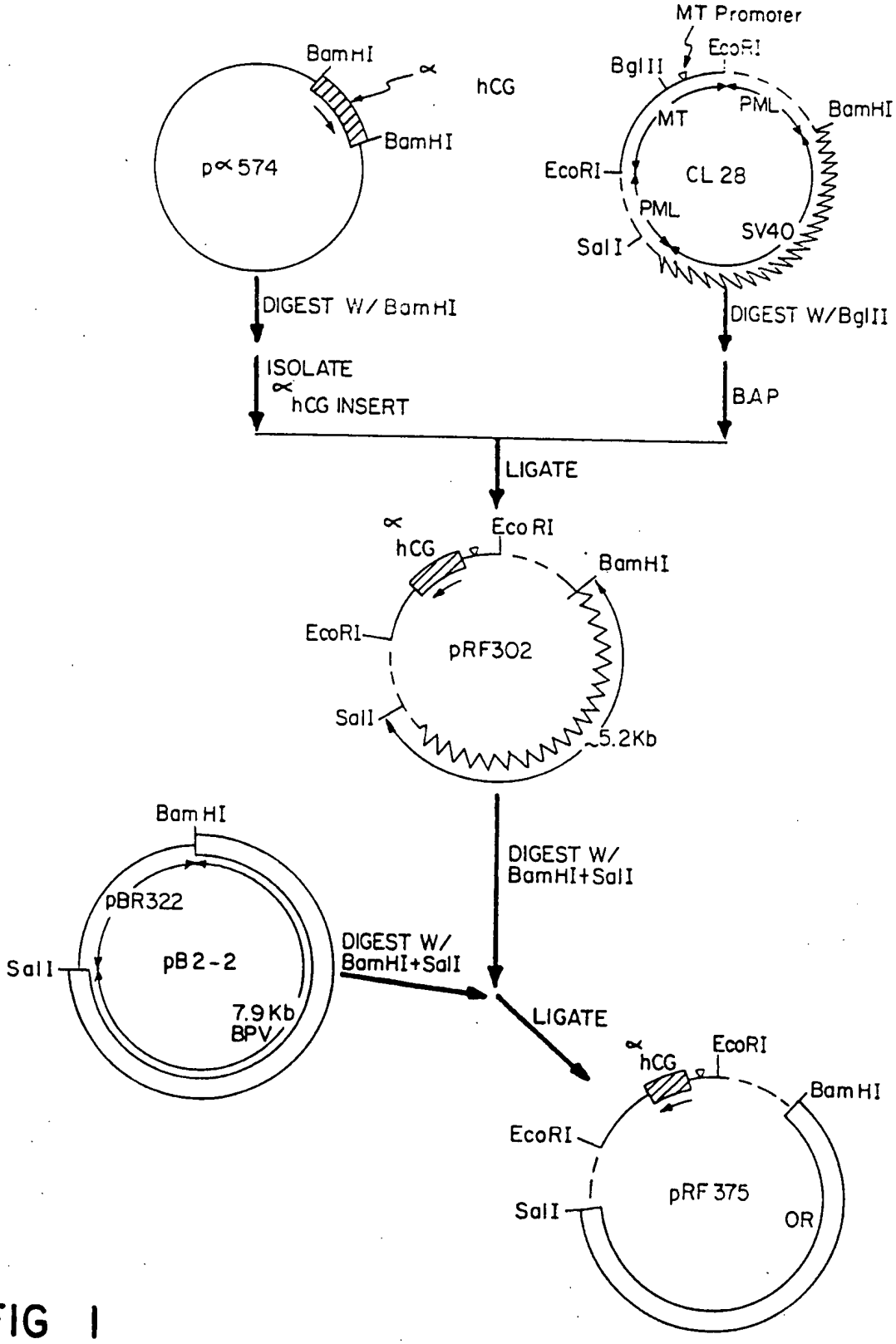


EXHIBIT A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BamHI
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DIGEST W/ BamHI
ISOLATE ∝ hCG INSERT
MT Promoter
EcoRI
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PML
BamHI
EcoRI
CL 28
PML
SV40
SalI
DIGEST W/BglII
BAP
LIGATE
∝ hCG
EcoRI
BamHI
EcoRI
pRF302
SalI
~5.2Kb
DIGEST W/ BamHI+SalI
BamHI
pBR322
SalI
pB2-2
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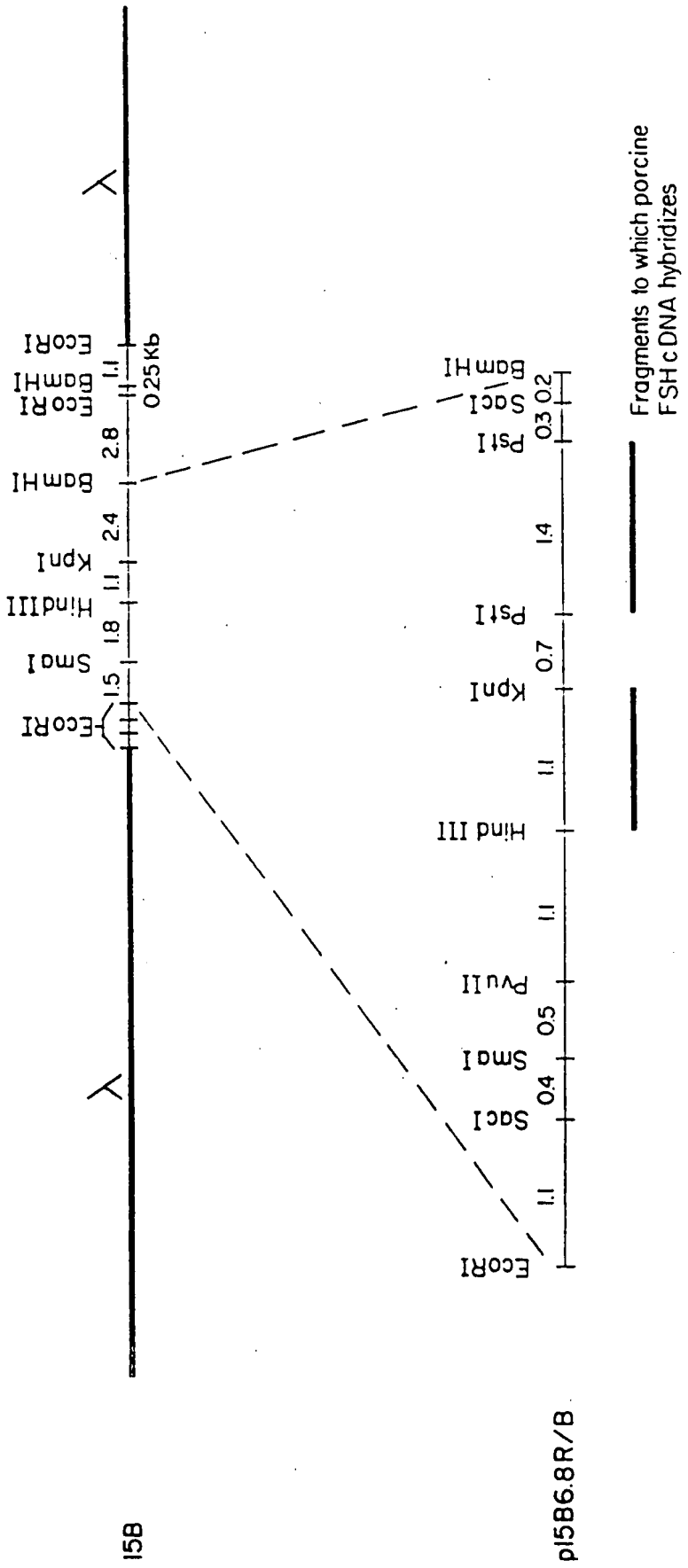
OR

FIG 1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2.4
1.1
1.8
1.5
λ
15B
BamHI
SacI
PstI
PstI
KpnI
Hind III
PvuII
SmaI
SacI
EcoRI
0.2
0.3
1.4
0.7
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1.1
0.5
0.4
1.1
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Fragments to which porcine FSH c DNA hybridizes

FIG 2

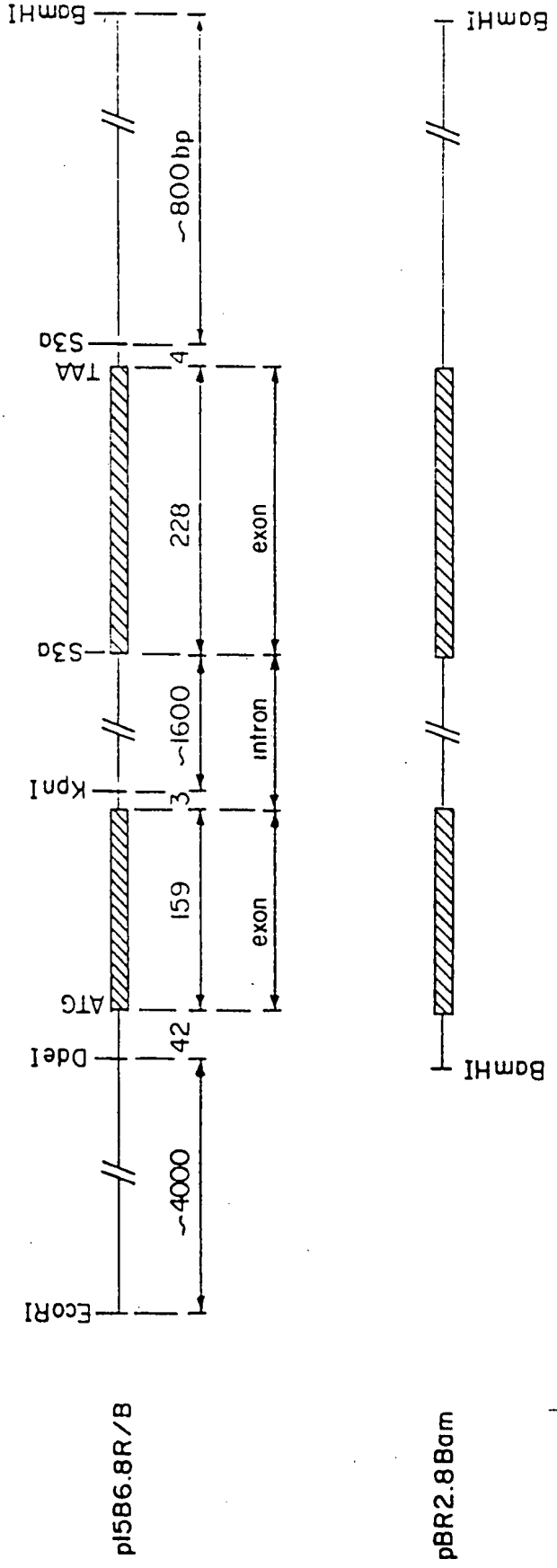
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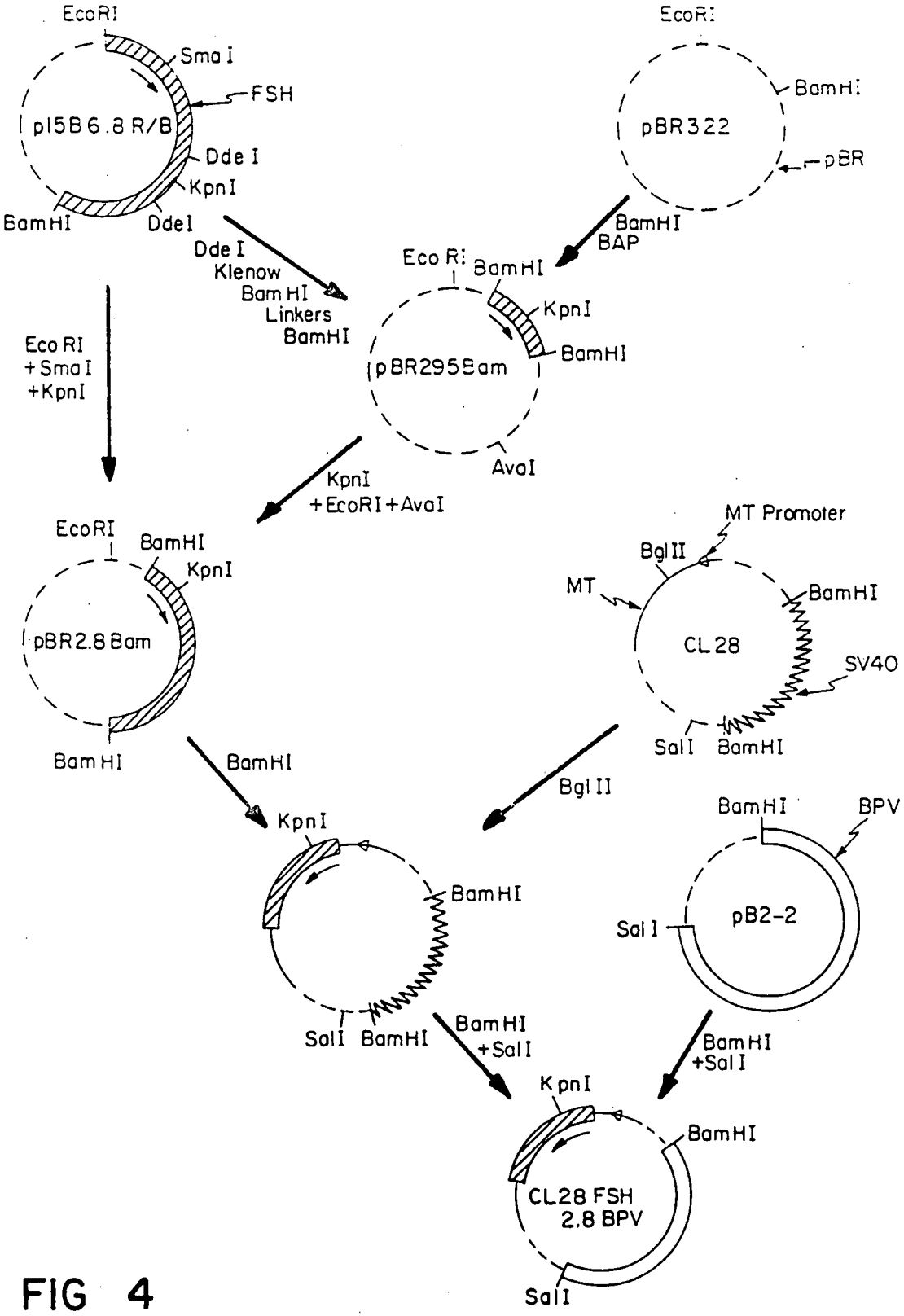
FIG 3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BamHI
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pBR
BamHI
BAP
Eco RI
BamHI
KpnI
pBR295Bam
BamHI
AvaI
Eco RI
+Sma I
+KpnI
KpnI
+EcoRI+AvaI
EcoRI
BamHI
KpnI
pBR2.8 Bam
Bam HI
BamHI
MT Promoter
BglII
MT
BamHI
CL28
SV40
SalI
BamHI
BglII
KpnI
BamHI
SalI
BamHI
BamHI
BPV
SalI
pB2-2
BamHI
+SalI
BamHI
+SalI
KpnI
BamHI
CL28 FSH
2.8 BPV
SalI

FIG 4

FOLLICLE STIMULATING HORMONE

This application is a continuation of application Ser. No. 696,647 filed on Jan. 30, 1985, now U.S. Pat. No. 4,923,805, which in turn is a continuation-in-part of application Ser. No. 548,228 filed Nov. 2, 1983, now U.S. Pat. No. 4,840,896.

BACKGROUND OF THE INVENTION

This invention relates to the use of recombinant DNA techniques to produce heteropolymeric proteins.

Various polypeptide chains have been expressed, via recombinant DNA technology, in host cells such as bacteria, yeast, and cultured mammalian cells. Fiddes, J. C. and Goodman, H. M. (1979) *Nature* Vol. 281, pg. 351-356 and Fiddes, J. C. and Goodman, H. M. (1980) *Nature* Vol. 286, pg. 684-687 describe the cloning of, respectively, the alpha and beta subunits of human choriogonadotropin (hCG).

Sugimoto U.S. Pat. No. 4,383,036 describes a process for producing hCG in which human lymphoblastoid cells are implanted into a laboratory animal, harvested from the animal, and cultured in vitro; accumulated hCG is then harvested from the culture.

SUMMARY OF THE INVENTION

In general the invention features the biologically active heterodimeric human fertility hormone follicle stimulating hormone ("FSH") which includes an alpha subunit and a beta subunit, each subunit being synthesized by a cell having an expression vector containing heterologuous DNA encoding the subunit.

The term "expression vector" refers to a cloning vector which includes heterologous (to the vector) DNA under the control of sequences which permit expression in a host cell. Such vectors include replicating viruses, plasmids, and phages. Preferred vectors are those containing at least the 69% transforming region, and most preferably all, of the bovine papilloma virus genome.

The invention permits the production of biologically active heterodimeric FSH from a single culture of transformed cells. The production of both subunits of FSH in the same cell eliminates the necessity of recombining subunits from separate cultures to assemble an active heterodimeric molecule. The system also allows production of FSH, in a single culture, which undergoes, in the culture, post-translational modification, e.g. glycosylation and proteolytic processing, for activity or stability.

In preferred embodiments, each expression vector is autonomously replicating, i.e., not integrated into the chromosome of the host cell. The use of autonomously replicating expression vectors prevents undesirable influence of the desired coding regions by control sequences in the host chromosome.

Other advantages and features of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

We turn now to the preferred embodiments of the invention, first briefly describing the drawings thereof.

DRAWINGS

FIG. 1 is a diagrammatic illustration of the construction of the plasmid pRF375.

FIG. 2 is a partial restriction map of the lambda clone 15B and the beta FSH-containing 6.8 kb EcoRI-BamHI fragment that is inserted into pBR322.

FIG. 3 is a partial restriction map of the beta FSH coding region and the BamHI fragment that is inserted into a BPV based expression vector.

FIG. 4 is a diagrammatic illustration of the construction of the BPV-containing plasmid CL28FSH2.8BPV, encoding the beta subunit of FSH.

STRUCTURE

The cloning vectors of the invention have the general structure recited in the Summary of the Invention, above Preferred vectors have the structures shown in the Figures, and are described in more detail below.

CONSTRUCTION OF CLONING VECTORS

Isolation of cDNA Clones Encoding the Common Alpha Subunit

In order to produce the heterodimeric FSH of the invention, the alpha subunit of human chorionic gonadotropin (hCG) first is isolated; the alpha subunit is common to the fertility hormones hCG, luteinizing hormone (LH), and FSH.

All of the techniques used herein are described in detail in Maniatis et al. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory), hereby incorporated by reference.

RNA is extracted from placental tissue by the following method. Homogenization of the tissue is carried out in a 1:1 mixture of phenol:100mM Na-acetate (pH 5.5) containing 1mM EDTA, that has been warmed to 60° C. for 20 min. After cooling on ice for 10 min., the phases are separated by centrifugation. The hot phenol extraction is repeated twice more followed by two extractions with chloroform.

RNA is precipitated from the final aqueous phase by the addition of 2.5 volumes of ethanol

In order to enrich for poly A+mRNA, placental RNA is passed over oligo (dT)-cellulose in 0.5M NaCl buffered with 10mM Tris-HCl, pH 7.5, and washed with the same solution. Poly A+mRNA is eluted with 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.05% SDS and precipitated twice with ethanol. Typical initial yields are 1.5-2.0 mg of total RNA per g of tissue, of which about 2% is poly A+mRNA.

Placental cDNA libraries are constructed by reverse transcription of placental mRNA, second strand synthesis using *E. coli* DNA polymerase I (large fragment), treatment with SI nuclease, and homopolymer tailing (dC) with terminal deoxynucleotidyl transferase; all such procedures are by conventional techniques.

In a typical preparation, 20-30% conversion of mRNA to single strand (ss) cDNA; 70% resistance to digestion with nuclease S1 after second strand synthesis; and dC "tails" of ten to twenty-five bases in length, are obtained. These cDNA molecules are then annealed to DNA fragments of the plasmid pBR 322, which has been digested with PstI, and to which dG "tails" have been added. These recombinant plasmids are then used to transform *E. coli* cells to generate a cDNA library (transformed cells are selected on the basis of tetracycline resistance).

In order to identify the human alpha hCG clone, a 219 bp fragment of a mouse alpha thyroid stimulating hormone (TSH) clone is used as a hybridization probe. This probe has 77% sequence homology with the human clone. It is radioactively labeled by nick translation and hybridized to the cDNA library under conditions that take into account the extent of homology. Strongly hybridizing clones are analyzed by restriction mapping and clones containing the complete coding sequence of alpha hCG are verified by DNA sequencing.

Construction of Plasmid pRF375

Referring to FIG. 1, the plasmid CL28 (identical to plasmid JYMMT(E); Hamer et al. (1983) J. Mol. Applied Gen. 1, 273-288), containing the murine metallothionein promoter, SV40 DNA, and pBR322 sequences, is cut with the restriction endonuclease BglII. At this site is inserted the cDNA clone of alpha hCG, containing untranslated regions of about 10 and 220 bp at its 5' and 3' ends, respectively. This clone has been genetically engineered by the addition of synthetic BamHI linkers at its termini.

The resulting plasmid pRF302 is digested with restriction enzymes BamHI and SalI to release the SV40 DNA sequence.

Plasmid pB2-2, which contains the entire BPV genome, and some pBR322 sequences, is digested with BamHI and SalI to yield the BPV genome with BamHI/SalI ends; this fragement is ligated into pRF302 containing the metallothionein-hCG sequences.

Following transformation of *E. coli,* plasmid pRF375 is identified and isolated. It encodes the common alpha subunit under the control of the mouse metallothionein promoter.

Isolation of the Human beta FSH Gene

A human genomic library in phage lambda (Lawn et al., 1978, Cell 15, p. 1157-1174) is screened using "guessed" long probes. The idea behind such probes, set forth in Jaye et al. (1983) Nucleic Acids Research 11(8), 2325, is that if the amino acid sequence of a desired protein is at least partially known, a long probe can be constructed in which educated guesses are mad as to the triplet encoding any amino acid which can be encoded by more than one, and not more than four, different triplets. Any correct guesses increase the amount of homology, and improve the specificity, of the results.

To isolate desired regions of DNA, two labeled 45-mer probes are used: TB36, homologous with amino acids 56-70 of human beta FSH; and TB21, homologous with amino acids 73-87. These probes have the following nucleotide compositions (corresponding amino acids are also given):

```
TB36:          Val—Tyr—Glu—Thr—Val—Lys—Val—
(AA56-70) 3'   CAC ATG CTC TGG CAC TCT CAC

               Pro—Gly—Cys—Ala—His—His—Ala—Asp
               GGT CCG ACG CGG GTG GTG CGA CTG 5'

TB21:          Tyr—Thr—Tyr—Pro—Val—Ala—Thr—
(AA73-87) 3'   ATG TGC ATG GGT CAC CGA TGT
```

-continued

```
Glu—Cys—His—Cys—Gly—Lys—Cys—Asp
CTC ACA GTG ACG CCG TTT ACG CTG 5'
```

The above probes are used to screen the human genomic library as follows. TB21 is labeled with ^{32}P and used to screen approximately 5×10, lambda plaques on duplicate filters by the in situ plaque hybridization technique of Benton and Davis (1977) Science 196, 180-182. The prehybridization solution is maintained at 55° C. for several hours and has the following composition: 0.75M NaCl; 0.15M Tris/HCl, pH 8.0; 10mM EDTA; 5 x Denhardt s Solution; 0.1% sodium pyrophosphate; 0.1% SDS; 100 microgram/ml *E. coli* t-RNA. The hybridization solution has the same composition except that it is maintained overnight at 45° C., and contains labeled probe in a concentration of about 0.5×10^6 cpm/ml. After hybridization, the filters are washed four times in $1 \times$SSC (=0.15M NaCl, 0.015M Na_3-citrate) and exposed to x ray film.

This screening procedure yields 50 plaques which hybridize to TB21 on both sets of filters. These 50 individual plaques are picked and combined into 10 culture pools containing 5 plaques each. The 10 cultures are grown and DNA is isolated from 50ml phage lysates. This DNA is then digested with EcoRI and fractionated on two identical 1% agarose gels, after which it is transferred to nitrocellulose paper according to the method of Southern (1975) J. Mol. Biol. 98, 503-517.

The DNAs on the two filters are hybridized to ^{32}p labeled TB21 and TB36, respectively. Individual plaques from the pool containing a restriction fragment which strongly hybridizes to both probes are then screened according to the above procedure, except that the DNAs are digested with EcoRI, BamHI, and EcoRI plus BamHI. In this way the 6.8kb EcoRI-BamHI fragment containing human beta FSH is isolated.

A partial restriction map of clone 15B, containing the 6.8kb EcoRI-BamHI fragment, is shown in FIG. 2. In order to locate the position of the beta FSH sequences within the clone, the 6.8 kb EcoRI-BamHI fragment of clone 15B is subcloned into pBR322 to yield plasmid p15B6.8R/B (FIG. 2). p15B6.8R/B is then digested with various restriction enzymes and the products are fractionated by agarose gel electrophoresis using conventional methods. The DNA is blotted to nitrocellulose paper and hybridized to fragments of a porcine beta FSH cDNA clone labeled with ^{32}p by nick translation.

As shown in FIG. 2, the porcine beta FSH probe hybridizes to only two fragments of the human DNA, namely a 1.1kb HindIII-KpnI and a 1.4kb PstI fragment Partial DNA sequencing of these two fragments shows that this DNA indeed codes for human beta FSH and that the entire coding region for beta FSH is contained in these two fragments.

As shown by the restriction map of FIG. 3, the beta FSH coding sequence is interrupted by an intervening sequence of approximately 1.6kb between amino acids 35 and 36 of mature beta FSH. The nucleotide sequence of the entire human beta FSH coding region and some of the flanking and intervening sequences are given below. The amino acid sequence deduced from the nucleotide sequence is given for the coding region.

```
                                                                30                  60
GCT TAC ATA ATG ATT ATC GTT CTT TGG TTT CTC AGT TTC TAG TGG GCT TCA TTG TTT GCT
```

-continued

```
                                                    90                                                  120
TCC CAG ACC AGG [ATG] AAG ACA CTC CAG TTT TTC TTC CTT TTC TGT TGC TGG AAA GCA ATC
                [Met] Lys Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile

                                                   150                                                  180
TGC TGC AAT AGC TGT GAG CTG ACC AAC ATC ACC ATT GCA ATA GAG AAA GAA GAA TGT CGT
Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys Glu Glu Cys Arg

                                                   210                                                  240
TTC TGC ATA AGC ATC AAC ACC ACT TGG TGT GCTGGC TAC TGC TAC ACC AGGGTA GGT ACC
Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly Tyr Cys Tyr Thr Arg

                                                   270                                                  300
// ATG TTA GAG CAA GCA GTA TTC AAT TTC TGT CTC ATT TTG ACT AAG CTA AAT AGG AAC

                                                   330                                                  360
TTC CAC AAT ACC ATA ACC TAA CTC TCT TCT TAA ACT CCT CAG GAT CTG GTG TAT AAG GAC
                                                                Asp Leu Val Tyr Lys Asp

                                                   390                                                  420
CCA GCC AGG CCC AAA ATC CAG AAA ACA TGT ACC TTC AAG GAA CTG GTA TAT GAA ACA GTG
Pro Ala Arg Pro Lys Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val

                                                   450                                                  480
AGA GTG CCC GGC TGT GCT CAC CAT GCA GAT TCC TTG TAT ACA TAC CCA GTG GCC ACC CAG
Arg Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val Ala Thr Gln

                                                   510                                                  540
TGT CAC TGT GGC AAG TGT GAC AGC GAC AGC ACT GAT TGT ACT GTG CGA GGC CTG GGG CCC
Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys Thr Val Arg Gly Leu Gly Pro

                                                   570
AGC TAC TGC TCC TTT GGT GAA ATG AAA GAA [TAA] AAA TCA GTG GAC ATT TC
Ser Tyr Cys Ser Phe Gly Glu Met Lys Glu [End]
```

Still referring to the above sequence, there is a box around the ATG initiation codon of the 18 amino acid signal peptide, which is cleaved post-translationally. The mature protein begins with the amino acid Asn encoded by the circled triplet AAT. The exon-intron boundaries are marked by arrows; they are flanked by the concesus sequence GT for the splice donor and AG for the splice acceptor site. There is a box around the stop codon TAA, the end of the coding region.

Below is a reproduction of the above sequence not broken into triplets, showing restriction sites; the ATG beginning and the TAA ending the coding region are boxed and the exon-intron junctions are marked by arrows.

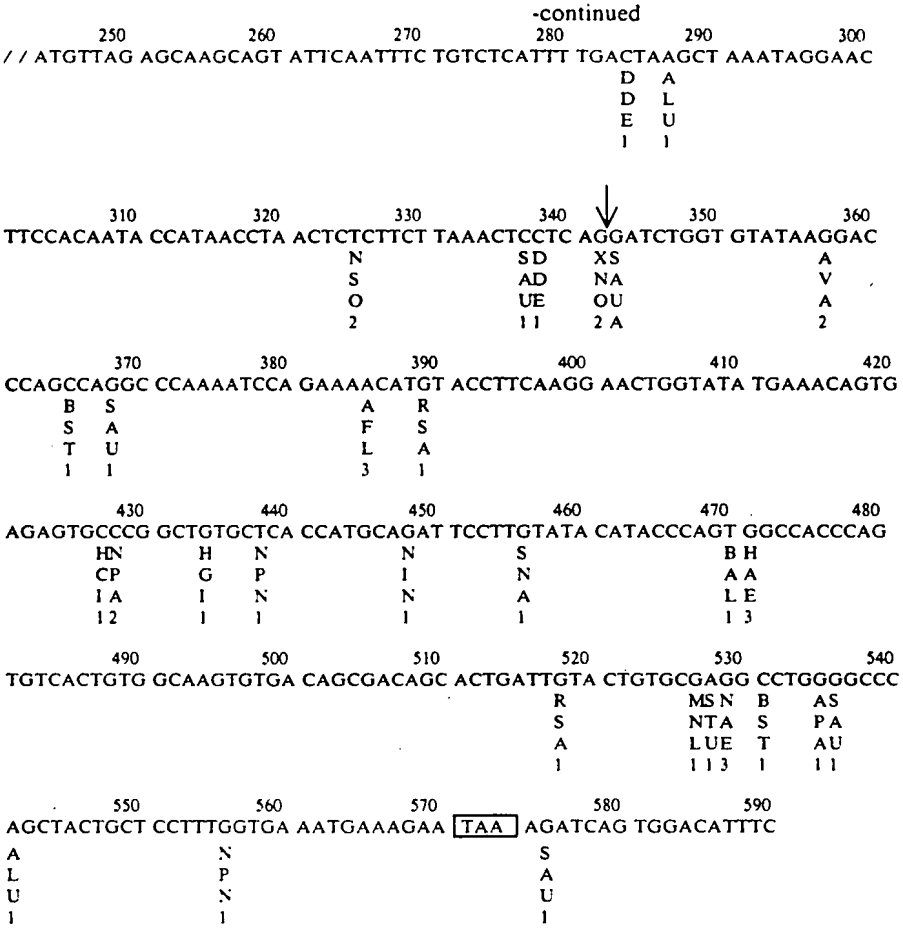
```
          10        20        30        40        50        60
GCTTACATAA TGATTATCGT TCTTTGGTTT CTCAGTTTCT AGTGGGCTTC ATTGTTTGCT
                                  D
                                  D
                                  E
                                  1

          70        80        90       100       110       120
TCCCAGACCA GG [ATG] AAGAC ACTCCAGTTT TTCTTCCTTT TCTGTTGCTG GAAAGCAATC
      B   F   N                 N
      S   O   B                 B
      T   K   O                 O
      1   1   2                 2

         130       140       150       160       170       180
TGCTGCAATA GCTGTGAGCT GACCAACATC ACCATTGCAA TAGAGAAAGA AGAATGTCGT
B         A         A         N                   N
B         L         L         P                   B
V         U         U         N                   D
1         1         1         1                   2

         190       200       210       220       230       240
TTCTGCATAA GCATCAACAC CACTTGGTGT GCTGGCTACT GCTACACCAG GGTAGGTACC
          S                                       R         KR
          F                                       S         PS
          A                                       T         NA
          1                                       1         11
```

-continued



Insertion of the Beta FSH DNA into a BPV-Based Expression Vector

Referring to FIG. 3, a synthetic BamHI linker is inserted at the DdeI site of p15B6.8R/B, which is located 42 nucleotides 5' of the ATG initiation codon. Referring to FIG. 4, p15B6.8R/B is digested with DdeI and treated with *E. coli* DNA polymerase (Klenow), after which it is ligated to synthetic BamHI linkers and digested with BamHI. The 295 bp fragment containing the first exon of FSH is isolated and cloned into the BamHI site of pBR322. The resulting plasmid pBR295Bam is digested with KpnI plus EcoRI plus AvaI and ligated to p15B6.8R/B which has been digested with KpnI plus EcoRI plus SmaI. The ligation mix is then used to transform *E. coli*, and the plasmid pBR2.8Bam containing the human beta FSH DNA sequence as a BamHI fragment is identified from among the transformants by restriction mapping.

As shown in FIG. 4, expression plasmid CL28FSH2.8BPV is prepared according to the same method used to prepare pRF375 (FIG. 1), except that the 2.8 kb BamHI fragment of pBR2.8Bam is used in place of the alpha hCG cDNA clone. Plasmid CL28FSH2 8BPV can be used to transform mammalian host cells using conventional methods, and human beta FSH can be isolated and purified.

Transfection of Mouse Cells

To produce heterodimeric FSH using a mixed transfection, five μg of each BPV plasmid, i.e., pRF375 (alpha subunit) and CL28FSH2.8BPV (beta FSH), are mixed and added 0.5 ml of a 250 mM CaCl, solution containing 10 μg of salmon sperm DNA as carrier. This mixture is bubbled into 0.5 ml 280 mM NaCl, 50 mM Hepes and 1.5 mM sodium phosphate. The calcium phosphate precipitate is allowed to form for 30–40 minutes at room temperature.

24 hours prior to transfection, 5×10^5 cells of mouse C127 cells (available from Dr. Dean Hamer, National Cancer Institute, NIH, Bethesda, MD) are placed in a 100 mm dish or T-75 flask. Immediately before adding the exogenous DNA, the cells are fed with fresh medium (Dulbecco's Modified Medium. 10% fetal calf serum). One ml of calcium phosphate precipitate is added to each dish (10 ml), and the cells are incubated for 6–8 hours at 37° C.

The medium is aspirated and replaced with 5 ml of 2 glycerol in phosphate buffered saline, pH 7.0 (PBS) for 2 minutes at room temperature. The cells are washed with PBS, fed with 10ml of medium, and incubated at 37° C. After 20–24 hours, the medium is changed and subsequent refeeding of the cells is carried out every 3–4 days. Individual clones are grown in T-25 flasks. After 7–21 days, cell clones can be transferred to larger flasks for analysis.

Deposits

The following, described above, has been deposited in the Agricultural Research Culture Collection (NRRL), Peoria, IL 61604:

CL28FSH2.8BPV in *E. coli*, NRRL B-15923

The following, described above, has been in the American Type Culture Collection, Rockville, MD:

pRF375 in C127 cells, ATCC CRL 8401.

Applicants' assignee, Integrated Genetics, Inc., acknowledges its responsibility to replace these cultures should they die before the end of the term of a patent issued hereon, and its responsibility to notify the ATCC and NRRL of the issuance of such a patent, at which time the deposits will be made available to the public. Until that time the deposits will be made available to the Commissioner of Patents under the terms of 37 CFR §1.14 and 35 USC §112.

USE

The transformed cell lines of the invention are used to produce glycosylated, biologically active heterodimeric human FSH, which is purified from the cells and/or their culture media using conventional purification techniques. FSH has a number of well-known medical uses associated with human fertility. For example, FSH can be used, alone or in conjunction with hCG or LH, to induce ovulation, or superovulation for in vitro fertilization.

In addition, heterodimeric FSH, or the beta subunit alone, can be used in diagnostic tests for fertility and pituitary functions.

FSH produced by recombinant cells has the advantage, compared to FSH obtained from natural sources, of being free from contamination by other human proteins, in particular other fertility hormones.

Other embodiments are within the following claims. For example, rather than producing heterodimeric FSH by culturing cells containing two separate expression vectors, one encoding the alpha subunit and the other encoding the beta subunit, DNA encoding both subunits can be included in the same expression vector.

We claim:

1. A mammalian cell comprising a transformed cell transformed by at least a first expression vector, said transformed cell being capable of producing a biologically active heterodimeric human fertility hormone comprised of an alpha subunit and a beta subunit, each said subunit being encoded in nature by a distinct mRNA, said hormone being human FSH, the alpha subunit of said hormone being encoded by said first expression vector and the beta subunit of said hormone being encoded by said first expression vector or by a second expression vector by which said transformed cell is also transformed, or progeny of said transformed cell containing the genetic information imparted by said vector or vectors.

2. A mammalian cell in accordance with claim 1, said first vector being a plasmid.

3. A mammalian cell in accordance with claim 1, the alpha and beta subunits of said heterodimeric hormone being encoded by said first expression vector.

4. A mammalian cell in accordance with claim 1, transcription of the alpha and beta subunits of said heterodimeric hormone being under the control of the mouse metallothionein promoter.

5. A mammalian cell in accordance with claim 1, said cell being a mouse cell.

6. A mammalian cell in accordance with claim 1, said first expression vector being autonomously replicating.

7. A mammalian cell in accordance with claim 1, wherein said beta subunit is encoded by a second expression vector, distinct from said first expression vector, said transformed cell also being transformed by said second expression vector.

8. A mammalian cell in accordance with claim 7, said second expression vector being autonomously replicating.

9. A method for producing the biologically active human fertility hormone FSH comprising culturing host mammalian cells in accordance with claim 1.

10. The method of claim 9, wherein said beta subunit is encoded by a second expression vector, distinct from said first expression vector, said transformed cell also being transformed by said second expression vector, and wherein each said expression vector is autonomously replicating.

* * * * *

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Art Unit: 184
REDDY, Vermuri B. et al	)	Examiner: Wang, G.
Serial No.: 07/323,665	)	Washington, D.C.
Filed: April 24, 1989	)	June 17, 1991
For: FSH	)	

TERMINAL DISCLAIMER

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Genzyme Corporation, having a principal place of business at 75 Kneeland Street, Boston, MA 02111 (hereinafter "Genzyme") is the assignee of the entire right, title and interest of the above-identified patent application by virtue of an assignment recorded March 30, 1990, at Reel 5268, Frame 0958. Genzyme hereby disclaims the terminal part of any patent granted on the above-identified application which would extend beyond the expiration date of the full statutory term of United States Patent No. 4,923,805, and hereby agrees that any patent so granted on the above-identified application shall be enforceable only for and during such period that the legal title to said patent shall be the same as the legal title to United States Patent No. 4,923,805, this agreement to run with any patent granted on the above-identified application and to be binding upon the grantor, its successors or assigns. Genzyme does not disclaim any terminal part of any patent

granted on the above-identified application prior to the expiration date of the full statutory term of United States Patent No. 4,923,805 in the event that it later expires for failure to pay a maintenance fee, is held unenforceable, is found invalid, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321(1), has all claims cancelled by a reexamination certificate, or is otherwise terminated prior to expiration of its full statutory term, except for the separation of legal title stated above. This terminal disclaimer is being made without waiver of petitioner's rights under 35 U.S.C. 156 which may be available to extend the term of any patent granted on the above-identified application beyond the date set by this terminal disclaimer (37 CFR 1.775(a)). A charge form for payment of the fee required by 37 CFR 1.20(d) is attached hereto.

Genzyme Corporation

By: Mark A. Hofer
Signature

Mark A. Hofer, Esq.
Typed Name of Signatory

Assistant Secretary
Vice President, General Counsel
Title

7/12/91
Date

BROWDY AND NEIMARK
419 SEVENTH STREET, N.W.
WASHINGTON, D.C. 20004

MAINTENANCE FEE STATEMENT

The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 10, "status" below.

If a maintenance fee payment is defective, the reason is indicated by code in column 10, "status" below. An explanation of the codes appears on the reverse of the Maintenance Fee Statement. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

ITM NBR	PATENT NUMBER	FEE CDE	FEE AMOUNT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,156,957	183	990	----	07/323,665	10/20/92	04/24/89	04	NO	PAID

If the "status" column for a patent number listed above does not indicate "PAID" a code or an asterisk (*) will appear in the "status" column. Where an asterisk (*) appears, the codes are set out below by the related item number. An explanation of the codes indicated in the "status" column and as set out below by the related item number appears on the reverse of the maintenance fee statement.

EXHIBIT C

ITM NBR	ATTY DKT NUMBER
1	IGHETERODIME

3. METHOD OF MANUFACTURE AND PACKAGING

3.1 DEVELOPMENT GENETICS

The same production line is used in the 50 L cell culture process as for the 11.5 L process. Therefore, all information provided in Volume 2, pp. 71-213 of the New Drug Application submitted on September 16th, 1993 remains fully applicable.

3.2 CELL BANK SYSTEM

Production of crude r-hFSH in 50 L bioreactors is carried out using the same A2 cell line as that used for cell culture in the 11.5 L bioreactors. Therefore, the same manufacturer's Working Cell Bank (WCB) may be used to seed 11.5 L or 50 L bioreactors. *Information provided on the cell bank system in Volume 2, pp. 225-236 of the NDA submitted on September 16, 1993 remains applicable.*

WCB N° 2 was established using a similar expansion scheme as that previously described for WCB N° 1 (NDA September 1993, Volume 2, pp. 225-226) except that the cryopreservation medium was DMEM:F12 (1:1) containing 4 mM L-Glutamine, 20% FBS and 10% DMSO. The Population Doubling Level achieved was 44.4, identical to WCB N° 1.

This section describes the establishment and characterisation of an Extended Cell Bank (A2 ExCB 2.2) derived from a 50 L cell culture run seeded from WCB#2.

The numbering of the ExCB provides the following information. The first digit in ExCB **2**.2 indicates that this ExCB is derived from a cell culture run initiated with WCB N° 2. The second digit in ExCB 2.**2** indicates the scale of the cell culture run from which it is derived, i.e. the 11.5 L scale = 1 and the 50 L scale = 2.

3.2.1 Preparation of the extended cell bank

The A2 ExCB was established on September 12, 1994 from a 50 L cell culture run designated B19-7503-105. The expansion scheme was as follows:

At the end of production, a 200 ml sample containing microcarriers with attached cells and cells in suspension was withdrawn from the bioreactor. Two 850 cm^2 roller bottles were each inoculated with 100 ml of cell suspension. Growth medium used was DMEM/HAM's F12 supplemented with 10% FBS. Cells were subcultured following every 2 - 2.5 population doublings four times in 850 cm^2 roller bottles. The ExCB was established approximately 12 PDL's post production

This ExCB, N° 2.2 consists of 64 vials, each containing 20 x 10^6 viable cells.

3. METHOD OF MANUFACTURE AND PACKAGING

3.1 DEVELOPMENT GENETICS

The host cell line used for the production of recombinant human follicle stimulating hormone (r-hFSH) is an anchorage dependent Chinese hamster ovary (CHO) cell line, isolated from the CHO-K1 line and deficient in dihydrofolate reductase (DHFR) activity (Urlaub and Chasin, 1980). The CHO cells were co-transfected with two plasmids, pHαDHFR and pHFSHβODC, each containing two functional genes; the former possessing the DHFR selectable marker and the α-hFSH gene, and the latter possessing the ornithine decarboxylase (ODC) selectable marker and the β-hFSH gene.

The genomic DNA fragments encompassing sequences for the α- and β-subunits of human FSH were isolated from a lambda Charon 4A gene library carrying DNA from cells obtained from human fetal liver. This library was screened on separate occasions with a complementary DNA (cDNA) probe derived from the α-subunit of human chorionic gonadotropin (αhCG) and with oligonucleotides complementary to the β-hFSH gene. The αhCG cDNA probe identified a phage carrying a 17 kilobase pair (kb) insert with the complete genomic α-hFSH gene. Likewise, the oligonucleotide probes enabled the selection of a phage carrying a 13 kb insert containing the genomic β-hFSH gene. Subfragments of 11 kb and 2 kb containing genes for the α-hFSH or β-hFSH, respectively were introduced into expression vectors containing selectable markers.

The α-hFSH genomic fragment was inserted into the expression vector pCLH3AXSV2DHFR downstream of and juxtaposed to the mouse metallothionein promoter. The endogenous α-subunit polyadenylation signal was used for 3' processing of the α-subunit transcript. This expression vector contained the mouse DHFR gene as a selectable and amplifiable marker. The co-transfecting expression vector contained the β-hFSH gene in place of the α-hFSH gene and the selectable ODC gene in place of the DHFR gene. Since the β-hFSH polyadenylation signal was removed during engineering, the SV40 early polyadenylation signal supplied by the vector was used for 3' processing of the β-hFSH subunit transcript.

Co-transfection was carried out using the calcium precipitation procedure followed with methotrexate (MTX) treatment to amplify the recombinant genes. Individual transfectants were screened for FSH production by assaying culture supernatants for the secreted hormone. The A2 transfectant was selected and used to establish the manufacturer's Master Cell Bank (A2 MCB) on the basis of its high productivity (15 pg/cell/24 hr), its stability over time and the quality of the molecules secreted.

3.1.1 Source materials

a. Isolation of the human α- and β-hFSH genes

i) Preparation of the human genomic library

High molecular weight DNA from human fetal liver was isolated and partially digested with *Hae*III and *Alu*I, and fragments in the size range of 18-25 kb were prepared by sucrose gradient centrifugation (Lawn *et al.*, 1978). Fragments were treated with *Eco*RI methylase and synthetic dodecameric *Eco*RI linkers were ligated at both ends. *Eco*RI fragments with a size of 15-20 kb were introduced into the *Eco*RI site of bacteriophage lambda Charon 4A (Blattner *et al.*, 1977). The DNA mixture was *in vitro* packaged and used to transfect *Escherichia coli* strain DP50*SupF* (Leder *et al.*, 1977). Approximately 1 x 10^6 *in vitro* packaged phages were amplified 10^6 fold by low density growth on agar plates to establish a permanent library of cloned human DNA fragments.

ii) Isolation of α-hFSH gene

Approximately 1 x 10^6 recombinant bacteriophages were plated out using the host strain DP50 (Leder *et al.*, 1977). Replicas of the plates were made on nitrocellulose filters. Since the glycoprotein hormones are a group of structurally related molecules which share a common α-subunit, sequence information from the cDNA clone of αhCG (Fiddes and Goodman, 1979) was utilized to construct suitable probes for the identification of a full-length α-hFSH genomic clone. Recombinant phage DNA bound to the filters was hybridized with a 621 base-pair (bp) *Hin*dIII fragment carrying the "full-length" αhCG cDNA. This probe was used to select a recombinant bacteriophage containing a total of 17 kb of human DNA and which has been designated lambda Ch4Aαg. The DNA insert was

further analyzed with restriction endonuclease enzymes, in combination with Southern blotting against specific probes for the 5' and the 3' ends of the gene, i.e. a 230 bp fragment containing sequences from the 5' untranslated region to codon 34 of the mature protein and a 76 bp fragment containing the most distal part of the 3' untranslated region, respectively. The precise locations of the introns were established by DNA sequencing. The α-subunit gene spans a total of 9.4 kb of human DNA, out of which approximately 700 nucleotides encode the mature transcript (Figure 3.1.1-A). This gene is composed of four exons interrupted by three intron sequences. The largest intron is 6.4 kb long and is located in the 5' untranslated region, 7 nucleotides before the ATG region, separating the leader sequence from the coding portion of the gene. A 1.7 kb intron splits the codon which corresponds to the sixth amino acid of the mature protein, whereas the last intron, 0.4 kb in size is located between codons 67 and 68 (Fiddes and Goodman, 1981).

iii) Isolation of β-hFSH gene

The library described above (5 x 10^5 phages) was screened by hybridization with two 45mer oligonucleotide probes designed, according to the principles of Jaye *et al.* (1983), from the partially known amino acid sequence of the β-hFSH subunit. The selected oligonucleotides, TB36 and TB21, were approximately 80% homologous with the complementary nucleotide sequences of amino acids 56-70 and 73-87, respectively (Figure 3.1.2) and had the following compositions:

TB36	56	Val	Tyr	Glu	Thr	Val	Arg	Val	Pro	Gly	Cys	Ala	His	His	Ala	Asp	5'
	3'	CAC	ATG	CTC	TGG	CAC	TCT	CAC	GGT	CCG	ACG	CGG	GTG	GTG	CGA	CTG	
TB21	73	Tyr	Thr	Tyr	Pro	Val	Ala	Thr	Glu	Cys	His	Cys	Gly	Lys	Cys	Asp	5'
	3'	ATG	TGC	ATG	GGT	CAC	CGA	TGT	CTC	ACA	GTG	ACG	CCG	TTT	ACG	CTG	

This screening procedure yielded 50 clones which hybridized with oligonucleotide TB21. Hybridization between the restriction endonuclease digested DNA of the selected clones and oligonucleotide TB36 allowed isolation of one clone, designated lambda 15B (Beck *et al.*, 1985), which contained 13 kb of human DNA.

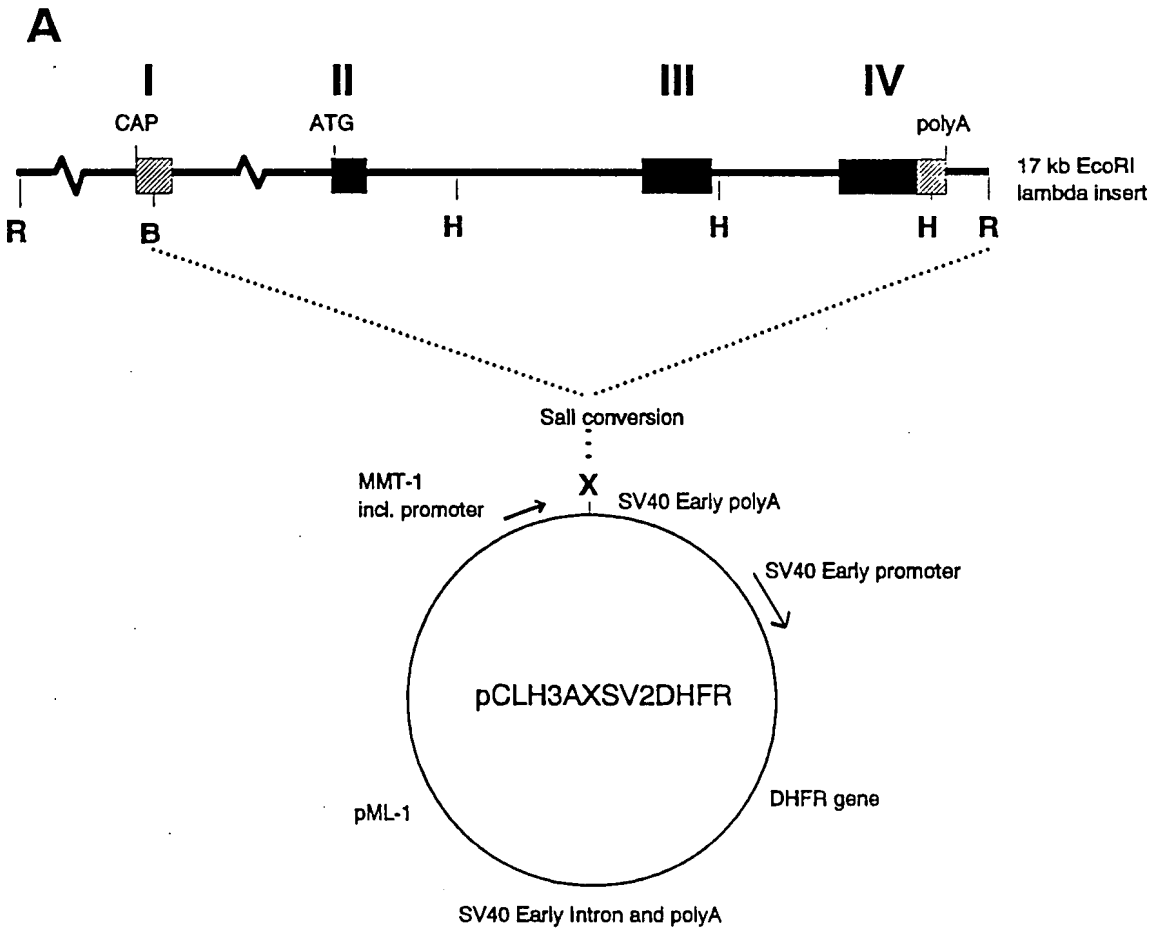


Figure 3.1.1-A: Structural organization of the transfection plasmid pHαDHFR derived from the insertion of the genomic DNA fragment encompassing the α-hFSH gene into the vector pCLH3AXSV2DHFR. The bold horizontal line depicts the 17 kb *Eco*RI human genomic DNA fragment containing α-hFSH gene sequences cloned into lambda Charon 4A. Boxes correspond to exons I to IV; translated and non-translated regions are shown as shaded and hatched, respectively. The predicted cap site (CAP), the initiation codon (ATG) and the approximate position of the polyadenylation site (poly A) are indicated. Restriction endonuclease sites are R (*Eco*RI), H (*Hin*dIII), and X (*Xho*I). The dotted lines show the extent of DNA sequences cloned into the *Xho*I site of the expression vector. Arrows indicate the direction of transcription from the MMT-1 and SV40 early promoters on the vector pCLH3AXSV2DHFR. The locations of DHFR, SV40 Early polyadenylation and pML-1 sequences are indicated.

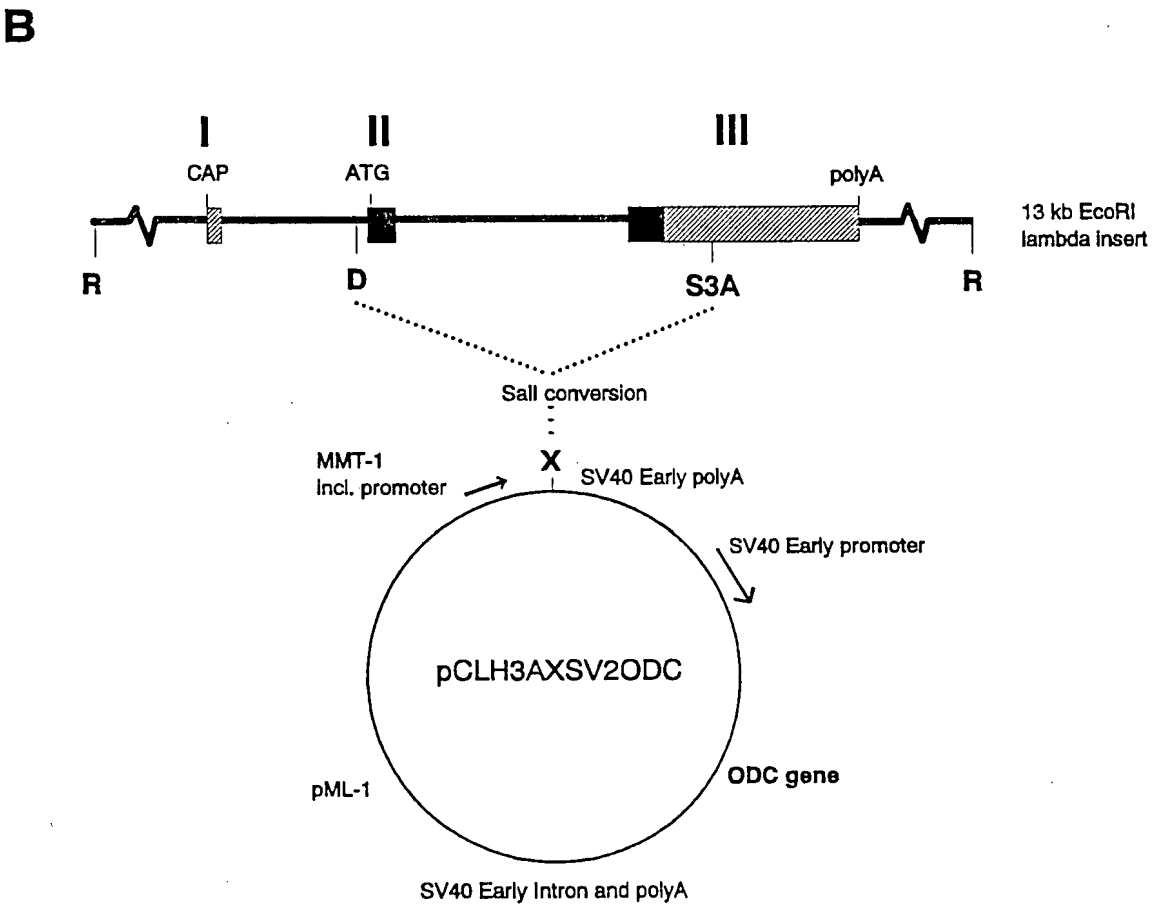


Figure 3.1.1-B: Structural organization of the transfection plasmid pHFSHβODC derived from insertion of the genomic DNA fragment encompassing the β-hFSH gene into the vector pCLH3AXSV2ODC. The bold horizontal line depicts the 13 kb *Eco*RI genomic DNA fragment containing β-hFSH gene sequences cloned into bacteriophage lambda Charon 4A. Restriction endonuclease sites are R (*Eco*RI), D (*Dde*I), S3A (*Sau*3A) and X (*Xho*I). The vector pCLH3AXSV2ODC is identical to pCLH3AXCV2DHFR (Figure 3.1.1a) except that the DHFR selectable marker has been replaced by a mouse ODC gene marker.

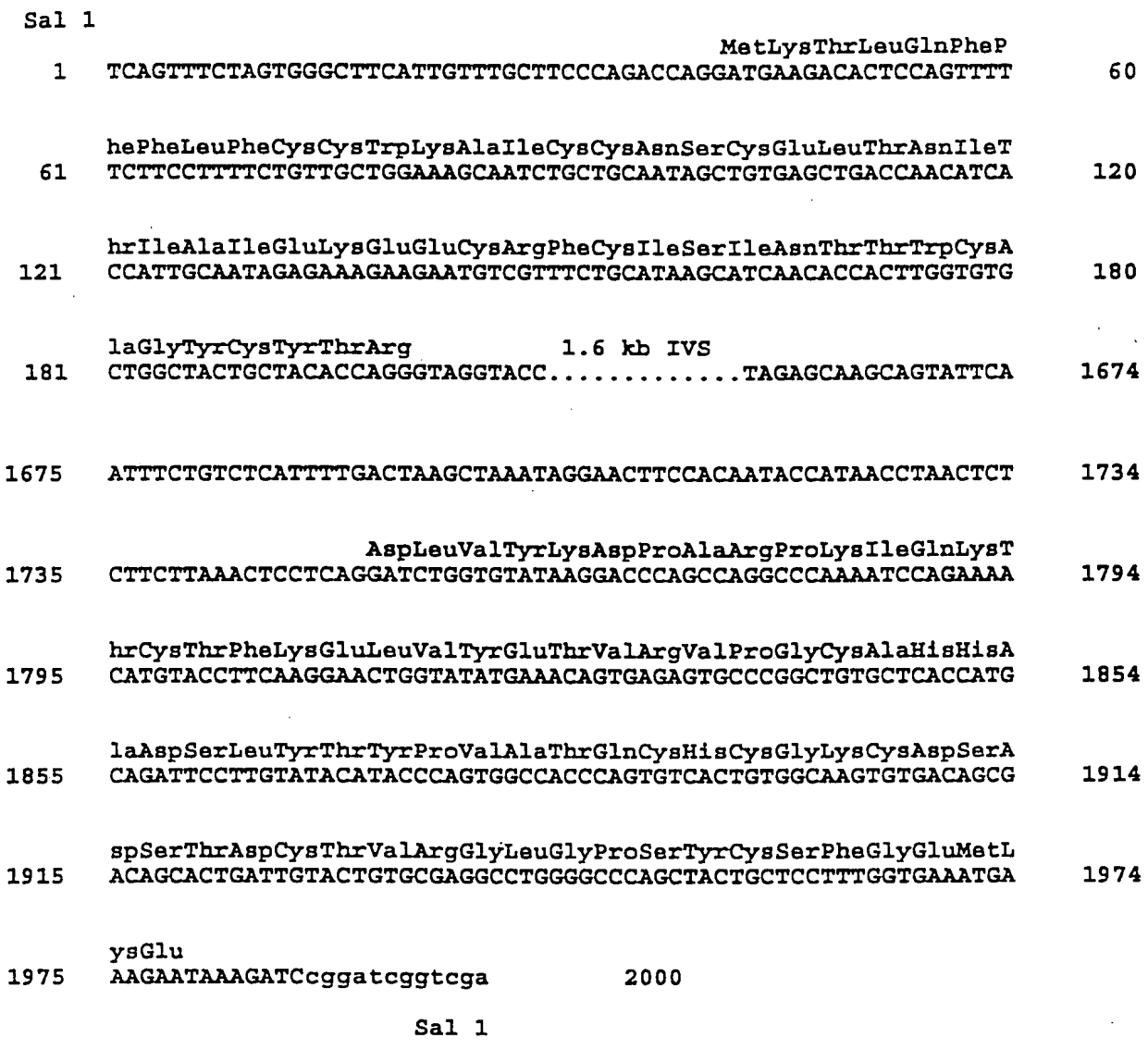


Figure 3.1.2: Partial sequence of the engineered 2 kb *Dde*I-*Sau*3A fragment containing the coding sequence of the β-hFSH gene. Nucleotides in lower case at the 3' terminus results from attachment of synthetic linkers. The β-hFSH amino acid sequence marks the coding regions of exons II and III. The dotted line represents uncharacterized intervening sequence (IVS).

A 6.8 kb *Eco*RI-*Bam*HI subfragment (Watkins *et al.*, 1987) derived from the 13 kb human genomic lambda insert was subcloned into pBR322 to yield plasmid p15B6.8R/B. Further Southern blot analysis of restricted p15B6.8R/B DNA showed that coding sequences were included within two fragments; 1.1. kb *Hin*dIII-*Kpn*I and 1.4 kb *Pst*I fragments. Partial sequencing showed that these two fragments encompassed the entire coding region for β-hFSH. The nucleotide sequence of the entire human β-hFSH coding region and some of the flanking and intervening sequences are given in Figure 3.1.2. The β-hFSH coding sequence is interrupted by an intervening sequence of approximately 1.6 kb between the codons for amino acids 35 and 36 of the β-hFSH. The exon-intron boundaries are flanked by the consensus sequence GT for the splice donor site and AG for the splice acceptor site. The location of this intron is strictly conserved in all the glycoprotein β-subunit genes examined to date (Jameson *et al.*, 1988). A second intron occuring 6 bp upstream of the translational start site and containing two alternate splicing donor sites which may give rise to 5'-untranslated sequences of 63 or 33 bases in length, has been recently described (Jameson *et al.*, 1988). These authors also showed that at least two polyadenylation sites can be used by the β-hFSH transcripts. The predicted translation product of this gene is a 129 amino acid long precursor including a typically hydrophobic 18 amino acid signal peptide.

b. Description of the host cell

A CHO cell line, designated DUKX-B11, which lacks DHFR (tetrahydrofolate dehydrogenase, 7,8-dihydrofolate: NADP+ oxidoreductase; EC 1.5.1.3) activity, was used as the host cell. The cell line was isolated from the CHO-Kl line (Kao and Puck, 1968) by mutagenesis with ethyl methanesulfonate followed by gamma irradiation. DHFR deficient mutants were selected by exposure to high specific activity [^{3}H] - deoxyuridine (Urlaub and Chasin, 1980). Full deficient mutants require glycine, hypoxanthine and thymidine for growth. The central role of DHFR in the synthesis of nucleic acid precursors, together with the sensitivity of DHFR deficient cells to tetrahydrofolate analogs such as methotrexate (MTX), present two major advantages. Firstly, transfection of such DHFR deficient cells by plasmids containing a DHFR gene allows the selection of recombinant MTX resistant cells.

Secondly, culture of these cells in selective media containing increasing concentrations of MTX results in amplification of the DHFR gene and the associated DNA (Kaufman and Sharp, 1982).

3.1.2 Preparation of the cell line

Anchorage dependent, DHFR deficient CHO cells (Urlaub and Chasin, 1980) were co-transfected by the calcium phosphate precipitation procedure with two plasmids, internally designated as pHαDHFR and (Figure 3.1.1-A) and pHFSHβODC (Figure 3.1.1-B), containing the selectable DHFR marker and genomic α-hFSH gene and the ODC marker and the β-hFSH gene, respectively. To amplify the transfected genes, selected cell lines were submitted to MTX treatment. Cell line 39 was subjected to limiting dilution cloning yielding transfectant A2. The steps used to construct and select the A2 transfectant are summarized in Figure 3.1.3.

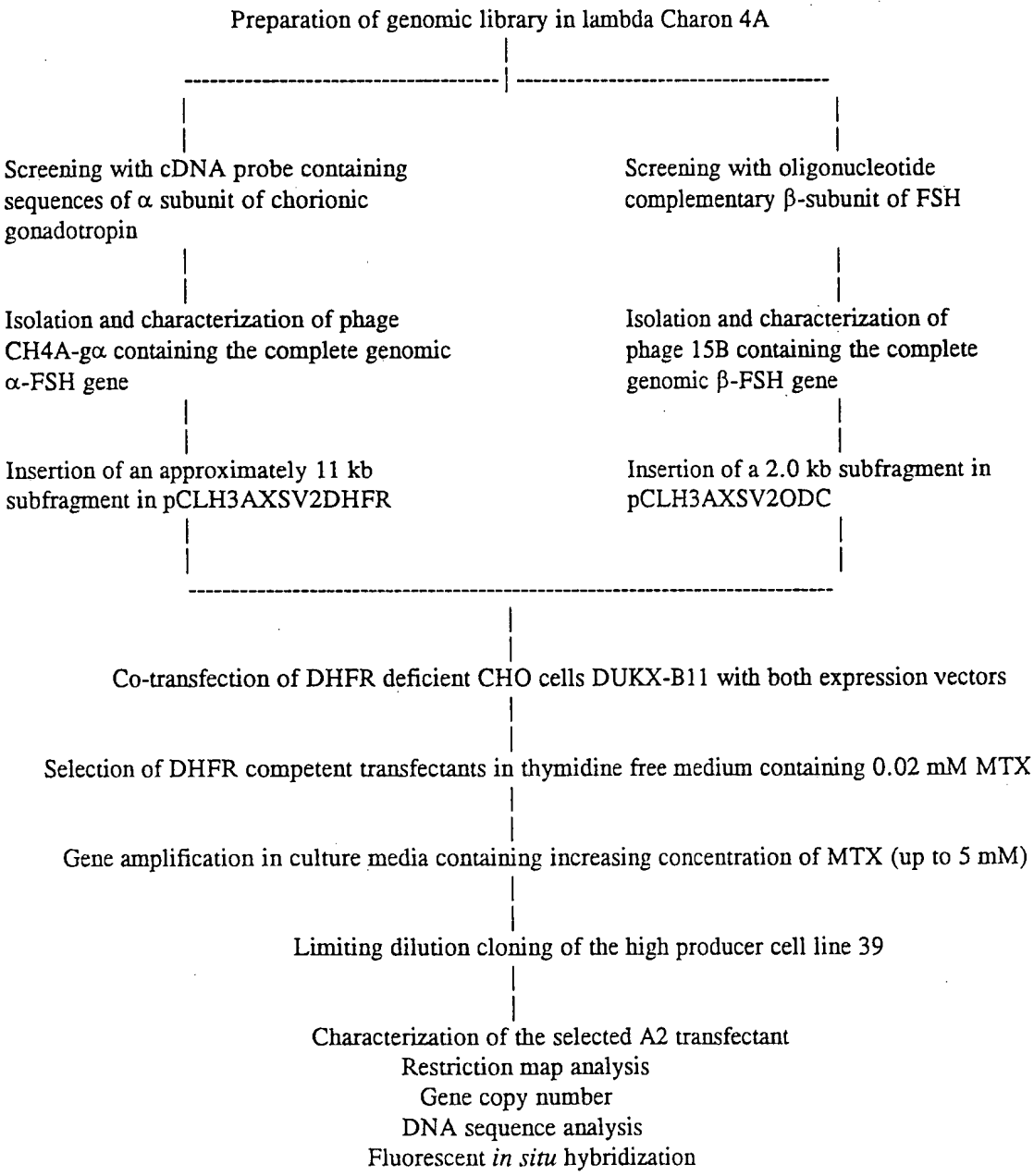


Figure 3.1.3: Outline of the construction and characterization of CHO cells expressing human FSH.

a. Construction of the expression plasmids

Detailed flow diagrams showing the DNA manipulation steps involved in the construction of the expression vectors pCLH3AXSV2DHFR and pCLH3AXSVODC are contained in Annex 3.1 A.

i) Expression vector carrying the α-hFSH gene

pCLH3AXSV2DHFR contains:

- a transcription unit derived from pSV2-*dhfr* (Subramani *et al.*, 1981) composed of the SV40 promoter (342 bp), the mouse DHFR cDNA (734 bp) and the small t intron and early polyadenylation signal (847 bp),
- 2616 bp from the plasmid pML-1 (Lusky and Botchan, 1981), derived from pBR322, carrying a bacterial ampicillin resistance gene and the ColEl bacterial origin of replication,
- 971 bp from the mouse metallothionein-I 3' flanking region (Hamer and Walling, 1982)
- 2000 bp from the mouse metallothionein-I gene promoter and 5' flanking region (Hamer and Walling, 1982) followed by the unique *Xho*I restriction endonuclease site,
- 242 bp containing the SV40 early polyadenylation region.

A *Bam*HI-*Eco*RI (3' terminal) subfragment of approximately 11 kb in size was derived from the α-genomic clone and contained part of exon I (less the first 35 nucleotides of the 5' untranslated region of the mRNA), exons II, III and IV, the intervening sequences and approximately 2 kb of 3' flanking sequence. This fragment was introduced, after conversion of both extremities to *Sal*I sites, into the unique *Xho*I restriction endonuclease site of pCLH3AXSV2DHFR (Figure 3.1.1-A). A detailed flow diagram showing the DNA manipulation steps involved in engineering the genomic α-gene fragment with flanking *Sal*I sites is contained in Annex 3.1 B (Figure 2).

The resulting recombinant plasmid, internally designated pHαDHFR, contained the genomic α-hFSH gene fused to the promoter of the mouse metallothionein-I gene. The genomic α-gene is followed by SV40 sequences carrying the early polyadenylation region. This plasmid contains also a DHFR gene used as a selectable and amplifiable marker.

ii) Expression vector carrying the β-hFSH gene

The pCLH3AXSV2ODC expression vector is similar to pCLH3AXSV2DHFR except that a 1.65 kb cDNA coding for mouse ODC (Chiang and McConlogue, 1988; Gupta and Coffino, 1985) was inserted between the HindIII and BglII sites of pCLH3AXSV2DHFR (Figure 3.1.1-A) in place of the DHFR cDNA.

A 2 kb *Dde*I-*Sau*3A subfragment containing the last 35 base pairs of the first intron, exon II, the protein coding region of exon III and the intron sequence which separates exons II and III of the β-hFSH gene was derived from the 6.8 kb *Eco*RI-*Bam*HI fragment of p15B6.8R/B. The *Dde*I and *Sau*3A were engineered for introducing *Sal*I restriction endonuclease sites at both extremities. The resulting fragment was inserted into the unique *Xho*I site of the expression vector pCLH3AXSV2ODC (Figure 3.1.1-B). Detailed flow diagrams showing the DNA manipulation steps involved in the plasmid construction are contained in Annex 3.1 B (Figures 4-6).

The resulting recombinant plasmid, internally designated pHFSHβODC, contained the genomic β-hFSH gene under the control of the mouse metallothionein-I gene promoter and a mouse ODC cDNA gene used as a selectable and amplifiable marker. ODC is the initial enzyme involved in the synthesis of polyamines, especially putrescine, which is an essential pathway for cellular growth. Cells (including wild-type CHO cells) cultured in media containing stepwise increases in concentration of ODC inhibitor, difluoromethylornithine, results in gene amplification similar to that observed with the DHFR marker.

b. Transfection

The DHFR-deficient cell line (CHO DUKX-B11) was cultured in Minimum Essential Medium (α-MEM) with ribonucleosides, supplemented with 2mM L-glutamine and

10% fetal bovine serum (FBS) at 37°C in 5% (α-MEM) CO_2. Cells were co-transfected using a modified calcium precipitation procedure (Graham and van der Eb, 1973) with uncut pHαDHFR containing the α-hFSH gene and pHFSHβODC containing the β-hFSH gene (molecular weight microgram: microgram ratio employed was 2 α-hFSH genes per β-hFSH gene). After six hours, cells were osmotically shocked by adding transfection solution containing 15% glycerol for 3.5 min. Cells were rinsed and re-fed with growth medium. After 48 hr, transfected cells were subcultured at a 1:10 split ratio and further cultured in selective medium consisting of α-MEM (without ribonucleosides and deoxyribonucleosides), supplemented with 10% dialyzed FBS and 0.02 μM MTX.

c. Isolation of a constitutive FSH producing recombinant transfectant

Individual transfectants able to grow in the above culture conditions were isolated after 10-14 days by trypsinization in small transfection chambers and cultured in individual wells of 24 well plates. Cells from approximately 100 foci were transferred into T25 flasks from the original selection medium, and after 7-10 days of culture, the concentration of MTX was increased to 0.1 μM. A similar procedure was followed for each subsequent stepwise increase in MTX. The steps used were 0.02-->0.1--> 0.5--> 1.0-->5.0 μM MTX. Accumulation of r-hFSH in the culture medium of these amplified transfectants was quantified in logarithmically growing cultures for a period of 24 hours. Production rates (pg/cell/24 hrs) were determined by radioimmunoassay (MAIA Clone FSH, Serono Diagnostics, Allentown PA).

It was observed that co-amplification of the α-hFSH and β-hFSH plasmids was a frequent occurrence when the cells were exposed to stepwise increases of MTX concentration. Similar results (co-integration and co-expression of transcription units carried on separate expression vectors) were obtained by Kaufman *et al.* (1985). This protocol selects recombinants wherein both recombinant plasmids are integrated into genomic sites and where gene amplification is efficient. Therefore direct and separate amplification of the β-hFSH plasmid using ODC antagonist was unnecessary.

Eight individual cell lines were isolated which displayed wild-type CHO cell morphologies with population doubling times ranging from 20.9 to 31.7 hours and saturation densities from 1 to 3 x 10^5 cells/cm^2, and they were able to produce (in

the presence or the absence of MTX) 4,500 to 94,000 IU/l of FSH (0.5 to 7.5 pg/cell/24 hrs). Cell line 39, exhibited the greatest degree of stability and the highest productivity values over an evaluation period encompassing 40 population doublings (6.14 pg/cell/24 hrs). In addition, preliminary physico-chemical characterization of the r-hFSH secreted in the culture medium showed that it exhibited *in vivo* activity in bioassays.

Cell line 39 was cloned by limiting dilution which involved plating 0.25, 0.5, 1, 2 or 4 cells per well (three 96-well culture trays per dilution). The dilution groups from which cell growth occurred in fewer than 33% of the wells in all three culture trays were chosen to obtain the lowest incidence of wells containing initially not more than one cell per well. As the culture became confluent, 93 transfectants were transferred from the 0.25 and 0.5 cell/well groups to 24-well culture plates. At confluence, the spent medium was removed and replaced with medium containing 1% FBS. After 48 hours, the medium from each well was sampled for r-hFSH and the cells from 40 individual transfectants were harvested and passed to one T25 flask. Similar expansion/evaluation steps were performed and 14 transfectants were further expanded in T75 flasks, and finally 8 in T150 flasks. On the basis of their r-hFSH production in medium containing 1% FBS, uniformity of morphology and ease with which they could be subpassaged, 3 of the 8 transfectants were selected and redesignated A1, A2 and A3. Accumulation of r-hFSH in the culture medium of these amplified transfectants was quantified by radioimmunoassay (MAIAclone) in stationary phase cultures following replacement of growth medium with a production medium containing 1% of dialyzed FBS. Their respective production rates were 3.3, 15.1 and 7.9 pg/cell/day (Table 3.1.1). The A2 transfectant was used to establish the master cell bank (MCB). A working cell bank (WCB) was established by expansion of cells recovered from a single vial of the MCB. Details on the preparation of cell banks are given in A. 3.2 'Cell bank system'.

Transfectant	FSH (IU/10^6 cells/day)	FSH (pg/cell/day)
A1	22.8	3.3
A2	106.0	15.1
A3	55.0	7.9

Table 3.1.1: Production rates of hFSHαGDbO#39 derived A1, A2 and A3 transfectants in T150 flasks.

3.1.3 Description of the cell line

a. Summary - Phenotypic and genotypic characterization

Cell growth kinetics, product yield, and general cellular morphology in T-flask cultures were selected as phenotypic characteristics suitable for comparative studies between cells derived from vials of the A2 master seed or master cell bank (A2 MCB) and the initial manufacturer's working cell bank (A2 WCB).

Cell growth kinetics were assessed by microscopic enumeration and vital staining, and quantified in terms of population doubling time (PDT) and the maximum cell yield (total cells per culture flask) obtained from a defined inoculum under standard culture conditions.

The results obtained confirmed the overall phenotypic consistency of A2 MCB and A2 WCB cells with respect to the parameters evaluated. No significant differences in cellular morphology were noted. Both cell banks exhibited similar growth patterns under these culture conditions. PDTs were 20.5 and 25.5 hours and maximum cell yields were 8.03 x 10^6 and 8.75 x 10^6 cells per T75 flask for the A2 MCB and A2 WCB, respectively. The volumetric productivity of hFSH (in units of hormone accumulated per litre of culture supernatant over a 24-hour period) by A2 WCB cultures was approximately 72% of that determined for cultures from the A2 MCB. The specific productivity (units of hormone produced per million cells per day) of A2 WCB cultures was determined to be 88% of that for A2 MCB cultures.

The apparent differences in total productivity observed between the two cell banks were due to differences in the numbers of cells per flask. When the specific cellular